

Mycophenolic acid suppresses granulopoiesis by inhibition of interleukin-17 production

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Mycophenolic acid is a commonly used immunosuppressant after organ transplantation and in autoimmune diseases; however, myelosuppression is a major complication despite its largely favorable side-effect profile. Mycophenolic acid targets inosine monophosphate dehydrogenase, which is essential for T-cell proliferation. The T-cell cytokine interleukin-17 (IL-17 or IL-17A) and its receptor maintain normal neutrophilic granulocyte numbers in mice by induction of granulocyte-colony-stimulating factor. To test whether mycophenolic acid induces neutropenia by inhibiting IL-17-producing T cells, we treated C57Bl/6 mice with mycophenolate-mofetil (the orally available pro-drug) and found a dose-dependent decrease in blood neutrophils. This myelosuppressive effect was completely abolished in mice that lack the IL-17 receptor. Mycophenolic acid delayed myeloid recovery after bone marrow transplantation and decreased the percentage of IL-17-producing T cells in the spleen and thymus, and inhibited IL-17 production in human and mouse T cells *in vitro*. Injection of IL-17 during mycophenolic acid treatment overcame the suppression of the circulating neutrophil levels. Our study shows that mycophenolic acid suppresses neutrophil production by inhibiting IL-17 expression, suggesting that measurement of this interleukin might be useful in estimating the risk of neutropenia in clinical settings.

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Myelosuppression is a common and potentially life-threatening side effect of immunosuppressive therapy. While some drugs such as cyclophosphamide or azathioprine directly and dose dependently affect myeloid cell proliferation and idiosyncratic neutropenia is a dose and time independent phenomenon not limited to immunosuppressives,¹ mycophenolic acid (MPA)-induced neutropenia is dose-dependent, but occurs typically more than 3 months after initiation of treatment. The incidence of leukopenia was 19% at a daily dose of 2 g and 38% at 3 g during 1 year in a large clinical trial of renal transplant patients.² A retrospective analysis of 78 liver-transplanted patients treated with the orally available prodrug mycophenolate mofetil showed a 17% incidence of neutropenia during a mean follow-up of 56 months.³ During maintenance immunosuppressive regimens after solid organ transplantation, rates of neutropenia ranged from 6 to 28% in 6- to 12-month observations.^{4–7} Plasma MPA is mostly albumin-bound and glucuronated to biologically active and inactive forms that can in turn alter MPA albumin binding and thereby biological activity.^{8–10} Despite association of lower drug levels with delayed graft function and rejection, and higher levels with toxicity, clinical trials have not yet shown a clinical benefit of therapeutic drug monitoring in renal transplant recipients.^{11,12}

In addition to being part of most standard regimens after organ transplantation,^{13,14} MPA is also used for treatment of autoimmune diseases including lupus nephritis^{15,16} and anti-neutrophil- cytoplasmic antibody-associated vasculitis.^{17,18} Therefore, understanding the mechanism of and possibly identifying the risk factors for developing neutropenia may become relevant for an extended cohort of patients.

MPA inhibits inosine monophosphate dehydrogenase (IMPDH), the rate-limiting enzyme in *de novo* guanosine biosynthesis that catalyzes the step from inosine monophosphate to xanthosine monophosphate.¹⁹ Two IMPDH isoforms are encoded by separate genes, *IMPDHI* and *IMPDHII*. MPA has a fivefold higher affinity for IMPDHII, which is upregulated in activated lymphocytes requiring *de novo* GMP synthesis.^{20,21} MPA has been shown to preferentially inhibit B- and T-lymphocyte proliferation.²² It does not affect the survival of T-lymphocytes in the G₁ phase of the cell cycle, but reduces the proliferation of both CD4⁺ and CD8⁺ T cells.²³ Addition of GMP, the final product of IMPDH, reverted these effects. MPA has differential effects on T-cell

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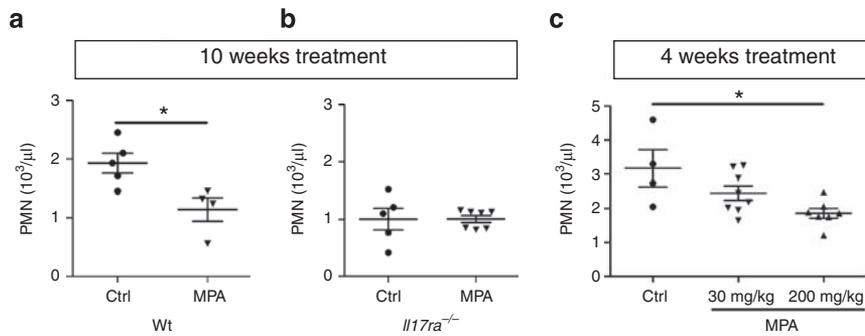


Figure 1 | MPA treatment depresses neutrophil counts in C57Bl/6 but not *Il17ra*^{-/-} mice. Eight-week-old wt C57Bl/6 were treated with 30 mg/kg/day mycophenolate (MPA) (triangles) or left untreated (circles) for 10 weeks. MPA treatment significantly decreased neutrophil counts (**a**) ($n = 4-5$, $*P < 0.05$). To test if the effect of MPA on neutrophil counts was related to IL-17, 7- to 9-week-old IL-17-receptor-deficient (*Il17ra*^{-/-}) mice were treated with 30 mg/kg/day MPA (triangles) or left untreated (circles) for 10 weeks (**b**), conducted in parallel with **a**) ($n = 4-7$). In a separate experiment, to test for dose response and short-term effects, 4-month-old female C57Bl/6 mice were treated with either 30 or 200 mg/kg/day MPA for 4 weeks (**c**). Neutrophil counts were significantly decreased after high-dose treatment ($n = 4-7$, $*P < 0.05$).

cytokine production. Pharmacological inhibition or genomic deletion of IMPDH did not change interleukin (IL)-2 or IL-2 receptor expression.²⁴ The T_{H1} signature cytokine interferon- γ (IFN- γ) was unchanged after IMPDH genomic disruption, but decreased in *ex vivo* models of pharmacological inhibition.^{25,26} Secretion of the T_{H2} marker cytokines IL-4 and IL-5 was reduced.²⁴

MPA has been studied in different animal models of inflammatory renal disease. MPA ameliorated experimental lupus nephritis in MRL/lpr mice,²⁷ mercury-induced glomerulonephritis²⁸ and Heymann nephritis in rats²⁹ where reduced IL-4 mRNA in immunization site draining lymph nodes and reduced mRNA expression of both IFN- γ and IL-4 in renal cortex mononuclear cells was found.

Granulocyte-colony-stimulating factor (G-CSF) is the major specific granulopoietic agent.³⁰⁻³² It is produced by a variety of cell types including bone marrow stromal and epithelial cells. However, T cells are critically involved in maintaining normal neutrophil blood counts.³³ Nude mice that lack thymic epithelium and therefore mature T cells have low neutrophil counts that were reconstituted by adoptive transfer of CD4⁺ T cells.³⁴ G-CSF is strongly induced by the T-cell cytokine IL-17, also known as IL-17A.^{35,36} Neutrophil counts were decreased by approximately 40% in mice lacking the IL-17 receptor (R)-A (*Il17ra*^{-/-}).^{37,38} Bone marrow regeneration after sub-lethal irradiation was impaired in mice lacking IL-17RA signaling.³⁹ Conversely, IL-17 overexpression resulted in marked neutrophilia.^{35,36} The effect of IL-17 on granulopoiesis is not direct, but requires secondary mediators, such as G-CSF produced by bone marrow stromal cells,⁴⁰ fibroblasts,⁴¹ and epithelial cells.^{42,43}

IL-17 is the signature cytokine of a novel T_H subset, T_{H17} cells that are vastly expanded in a number of autoimmune diseases.⁴⁴ IL-17 is also made by $\gamma\delta$ ⁺ and CD4⁻ $\alpha\beta$ ^{low} T cells in normal mice.^{38,45} IL-17 production is induced by a combination of IL-6 and transforming growth factor- β (TGF- β) and maintained by IL-23.⁴⁶⁻⁴⁹ IL-17-producing

T cells have been implicated in pathological inflammation.^{44,50,51} However, IL-17 is also required in host defense and acts largely by inducing neutrophil recruitment.⁵²⁻⁵⁴

The effect of MPA on IL-17 producer polarization has not been investigated. Given that MPA acts on proliferating T cells, we reasoned that the MPA effect on granulopoiesis might be mediated by inhibition of T-cell proliferation, causing IL-17 producer depletion over time resulting in reduced G-CSF and decreased granulopoiesis. We therefore investigated the effect of MPA on IL-17-producing T cells *in vitro* and *in vivo*.

RESULTS

MPA dose dependently decreases circulating neutrophil counts in mice

To test whether MPA reduced blood neutrophil counts in mice similar to that in humans and rats,⁵⁵ we treated C57Bl/6 mice with the clinical dose of 30 mg/kg for 10 weeks. This significantly reduced circulating blood neutrophil counts (Figure 1a). To test whether reduced neutrophil counts during MPA treatment were attributable to the IL-17 pathway, we used mice deficient in the main IL-17 receptor-A (*Il17ra*^{-/-}). As reported previously,^{37,38} *Il17ra*^{-/-} mice have reduced neutrophil counts (Figure 1b). Their level of neutrophils was unaffected by MPA treatment (30 mg/kg) for 10 weeks (Figure 1b). In a separate experiment using wild-type (wt) mice, a trend toward reduced neutrophil counts was already evident at 4 weeks of MPA administration at 30 mg/kg (Figure 1c). With this short-term treatment, a higher dose of MPA (200 mg/kg) significantly reduced blood neutrophil counts (Figure 1c). Both treatments were well tolerated (Supplementary Figure 1a and b). Concerning other hematopoietic cell lines, lymphocyte counts were decreased as expected in the peripheral blood and there also was a slight but significant decrease in erythrocyte counts (Supplementary Figure 1c and d). These data show that MPA treatment reduces blood neutrophil counts in normal mice, and that this effect requires IL-17 receptor-A.

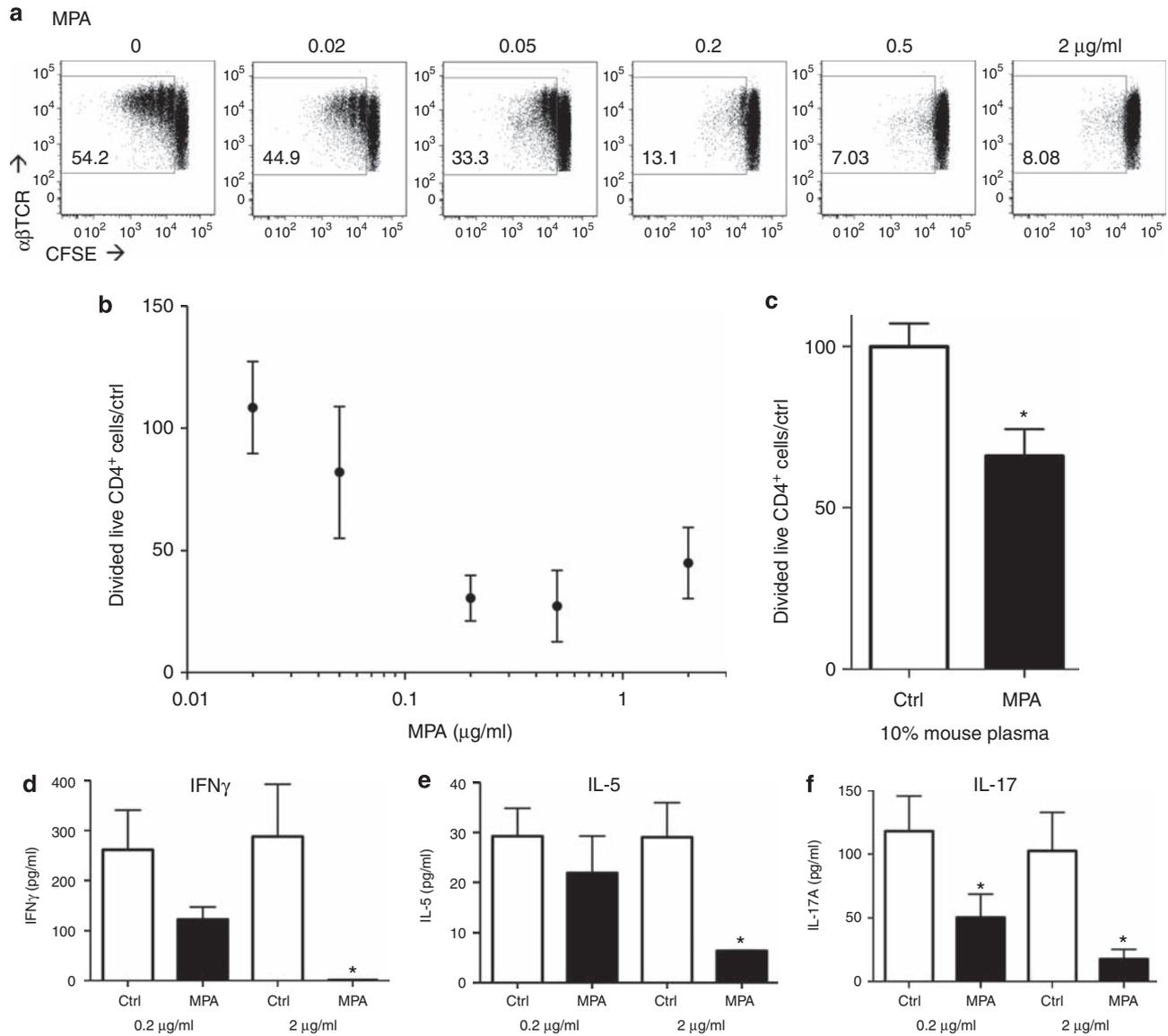


Figure 2 | MPA inhibits T-cell proliferation *in vitro* and *ex vivo*. Total splenocytes were stained with CFSE, treated with MPA or buffer control, and proliferation among live, CD4⁺ cells was assessed after 3 days on anti-CD3 and anti-CD28 (**a**, a typical example and **b**, means of three independent experiments). (**c**) 10% mouse plasma from MPA-treated or control mice was added to the culture and proliferation was assessed as in panel **a**. The T_{H1} marker cytokine IFN- γ (**d**), the T_{H2} marker cytokine IL-5 (**e**), and the T_{H17} marker cytokine IL-17 (**f**) were assessed in the supernatants of splenocytes cultured on anti-CD3 and anti-CD28 for 3 days (dimethylsulfoxide control versus 2 and 0.2 µg/ml MPA, $n = 4$) (* $P < 0.05$). CFSE, carboxyfluorescein-succinimidyl-ester.

MPA inhibits T-cell proliferation and polarization to IL-17 production *in vitro*

We next studied the dose response of MPA-mediated inhibition of T-cell proliferation and cytokine production. Total splenocytes were stained with carboxyfluorescein-succinimidyl-ester (CFSE) and stimulated with anti-CD3 and anti-CD28 for 3 days. Treatment with MPA resulted in a dose-dependent decrease in CD4⁺ T-cell proliferation (Figure 2a and b). To test if the mouse oral treatment regimen resulted in sufficient plasma levels to inhibit T-cell proliferation, 10% MPA-treated (30 mg/kg per day) or control mouse plasma was added to the culture media. Plasma from MPA-treated mice significantly reduced T-cell proliferation as measured by CFSE dilution (Figure 2c). The inhibition observed after addition of 10%

MPA-treated mouse plasma corresponded to 0.16 ± 0.09 µg/ml exogenous MPA and thereby a plasma concentration of 1.6 µg/ml after $10 \times$ dilution. Previous reports have shown that MPA can inhibit the production of a number of T-cell cytokines such as the T_{H1} marker cytokine IFN- γ ²⁶ and the T_{H2} marker cytokines IL-4 and IL-5.²⁴ To test how these were inhibited under non-lineage-specific T-cell stimulation (anti-CD3/anti-CD28) as compared with IL-17, cells were restimulated with phorbol-12-myristate-13-acetate (PMA)/ionomycin and supernatants were assessed for IFN- γ , IL-5, and IL-17 (Figure 2d-f). IL-17 production was significantly inhibited at 0.2 µg/ml MPA, when there was only a trend toward decreased IFN- γ , whereas significant IFN- γ and IL-5 inhibition required 2 µg/ml MPA, suggesting higher sensitivity of IL-17 producers to MPA.

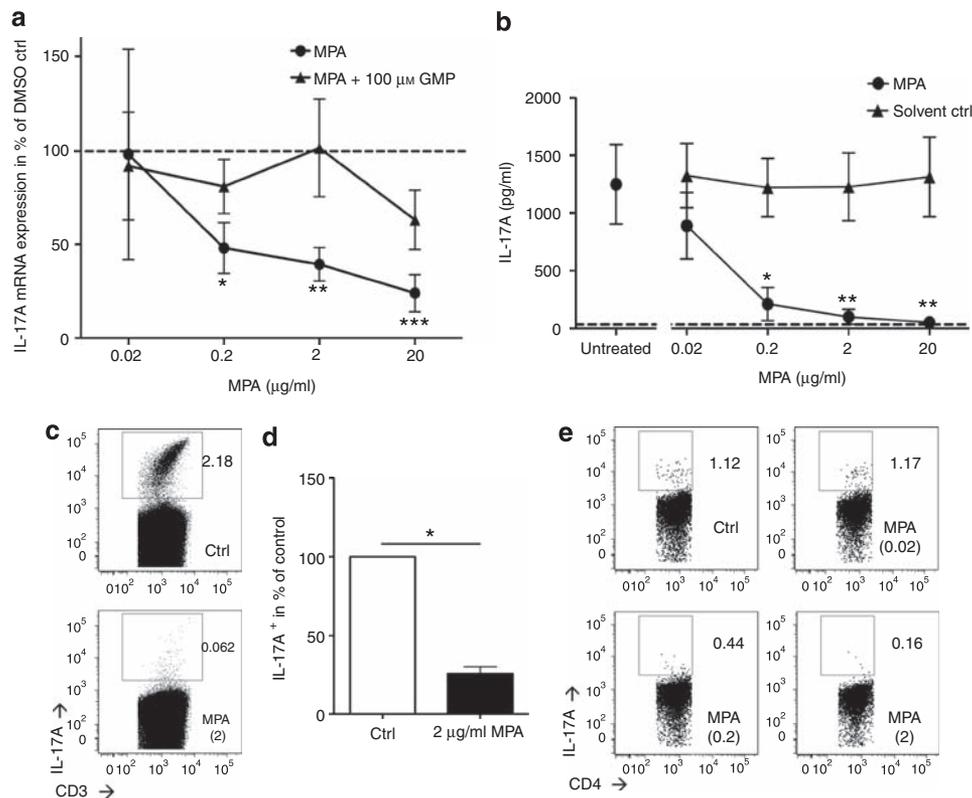


Figure 3 | MPA inhibits *in vitro* polarization of IL-17-producing T cells. Total splenocytes were polarized toward IL-17 production using IL-6, TGF- β , and IL-23 on anti-CD3 and anti-CD28. After 20 h, IL-17A mRNA was assessed with and without addition of 100 μ M GMP (**a**) (quantitative real-time PCR compared with β -actin, values in % of dimethylsulfoxide control, $n = 5-6$). After 3 days of stimulation, cells were transferred to an untreated plate, rested for 3 days, and re-stimulated with PMA/ionomycin for 5 h. IL-17A secretion into the supernatant was measured by ELISA (**b**, $n = 4$). The percentage of IL-17 producers among live CD3⁺ cells was assessed by flow cytometry (**c**) and quantified (**d**, $n = 5$) (* $P < 0.05$, ** $P < 0.01$). To test whether this effect was directly exercised on T cells, CD4⁺ splenocytes were isolated and treated with MPA (0.02, 0.2, and 2 μ g/ml). IL-17 production was analyzed by flow cytometry after intracellular staining (**e**, a typical of three independent experiments).

To more specifically investigate the effect of MPA on IL-17 production, we cultured wt mouse splenocytes under IL-17-polarizing conditions with IL-6, TGF- β , and IL-23.^{38,46} IL-17A mRNA was assessed by real-time reverse transcription-PCR after 20 h culture (Figure 3a). MPA dose dependently reduced IL-17A mRNA. To test for specificity, GMP, the product of IMPDH, the enzyme inhibited by MPA, was added. IL-17 mRNA expression was restored by GMP. To test if decreased mRNA resulted in decreased IL-17 protein expression, a 6-day standard protocol (three days of IL-6, TGF- β , and IL-23 treatment with stimulation on anti CD3/anti-CD28 followed by 3 days of treatment with IL-6, TGF- β , and IL-23 only) was used.^{38,46} On day 6, cells were re-stimulated by PMA/ionomycin and IL-17 secretion into the supernatant was measured by ELISA (Figure 3b). MPA dose dependently reduced IL-17 secretion. To investigate IL-17 biosynthesis at the single-cell level, we measured intracellular IL-17 after 6 days of IL-6, TGF- β , and IL-23 treatment (Figure 3c). On average, the number of IL-17-producing cells was reduced by 74% upon MPA treatment (Figure 3d). To test whether MPA directly affected IL-17-producing CD4⁺ T cells or acted indirectly through another cell type, the experiment was repeated in sorted CD4⁺ cells.

Flow cytometry of polarized CD4⁺ cells stained for intracellular IL-17 again showed a dose-dependent decrease of IL-17-producing cells by MPA treatment (Figure 3e).

To test if these results were also applicable to human lymphocytes, human peripheral blood mononuclear cells were polarized toward IL-17 production by IL-6 and TGF- β .⁵⁶ Flow cytometry after intracellular staining showed a significant decrease when MPA (2 μ g/ml) was added to the culture medium (Figure 4a and b). No such decrease was observed when the culture media was supplemented with GMP (Figure 4c). Tissue culture supernatants were examined for secreted IL-17A by ELISA and again there was a significant decrease in MPA-treated cells' media (Figure 4d) that was no longer present after addition of GMP (Figure 4e).

These data show a dose-dependent and IMPDH-specific effect of MPA on IL-17 production from mouse and human lymphocytes.

MPA inhibits neutrophil recovery and differentiation of IL-17-producing splenocytes *in vivo*

To test the effects of MPA on IL-17 producer differentiation *in vivo*, we reconstituted lethally irradiated mice with autologous bone marrow and subjected them to 4 weeks of

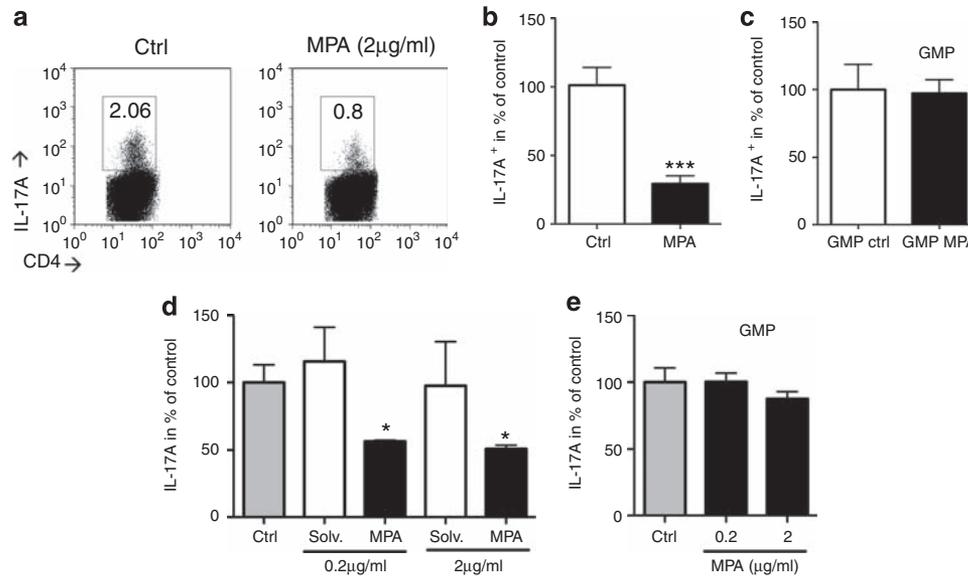


Figure 4 | Mycophenolate inhibits polarization of human IL-17-producing T cells. Human peripheral blood mononuclear cells were polarized toward IL-17 production using IL-6 and TGF- β . IL-17 production in live CD4⁺ cells was assessed by flow cytometry after intracellular staining (a). (b) Shown is the mean reduction in IL-17-producing cells by addition of 2 μ g/ml MPA from $n = 5$ donors ($***P < 0.001$). When GMP was added to the culture medium no reduction in the percentage of IL-17 producers was observed (c, $n = 5$). IL-17 production was also studied by ELISA of tissue culture supernatants. There was a significant reduction by MPA at concentrations of 0.2 and 2 μ g/ml (d, $n = 3$, $*P < 0.05$). No such decrease was observed in the presence of GMP (e, $n = 3$).

MPA treatment or left them untreated. As expected, MPA induced peripheral blood lymphopenia (Supplementary Figure 2a) and also decreased the peripheral blood red cell but not the thrombocyte counts (Supplementary Figure 3a and b). MPA suppressed blood neutrophil counts at 2 weeks of reconstitution (Figure 5a). There was no evidence of morphological changes in blood neutrophils by MPA treatment (Supplementary Figure 3a). Bone marrow cellularity in MPA-treated mice was decreased (Supplementary Figure 4b), but little change in bone marrow granulocyte Gr1 or 7/4 expression levels was observed (Supplementary Figure 4a and b).

IL-17 production in the splenocytes of the bone marrow-transplanted mice was assessed by flow cytometry (Figure 5b; total cell numbers are given in Supplementary Figure 2b). As described previously,⁴⁵ most IL-17A producers among CD3⁺ splenocytes were not CD4⁺, but rather CD4^{low} $\alpha\beta$ -TCR^{low}. Their number among all splenocytes was reduced by 60% at 2 weeks and by 31% at 4 weeks in splenocytes from MPA-treated mice (Figure 5c). Similar results were found in thymocytes (Supplementary Figure 2c and d). This resulted in a significant decrease of the total number of IL-17-producing cells per organ. These data suggest that MPA inhibits the recovery of IL-17-producing T cells after bone marrow transplantation.

Treatment with IL-17 normalizes circulating neutrophil numbers in MPA-treated mice during hematopoietic recovery

To test whether depression in circulating neutrophil counts by MPA was indeed due to lack of IL-17 rather than direct effects, for example, on myeloid progenitors or G-CSF-producing cells, we assessed whether IL-17 can reconstitute

circulating neutrophil counts in MPA-treated mice. MPA-treated bone marrow-transplanted mice were intraperitoneally injected with 1 μ g IL-17 or phosphate-buffered saline control on day 10 after bone marrow transplantation. This resulted in increased blood neutrophil counts at day 14 (Figure 6a). IL-17-induced rescue of blood neutrophil counts was associated with elevated plasma G-CSF levels in the same mice (Figure 6b). No significant change in circulating lymphocyte and erythrocyte levels was observed after injection of IL-17 (Supplementary Figure 5c and d). To test if IL-17 could also normalize circulating neutrophil levels in otherwise unchallenged mice treated with MPA, mice were treated for 4 weeks and then received an intraperitoneal IL-17 injection. This significantly increased circulating neutrophil counts after 3 days (Figure 6c). These results support the notion that MPA-mediated neutropenia is due to a defect in IL-17-producing T cells while downstream mediators can respond sufficiently to IL-17.

The bone marrow produces G-CSF in response to IL-17 stimulation with the highest expression of IL-17 receptor on CD115⁺CD11b⁺ monocytes

IL-17 induces G-CSF production from a number of tissues and cell types, including bone marrow stromal cells,⁴⁰ fibroblasts,⁴¹ and epithelial cells.^{42,43} Under homeostatic conditions *in vivo*, it has not been determined which organ or cell type is the main G-CSF producer. To start investigating this question, we first stimulated cell suspensions from a number of different organs (spleen, mesenteric lymph nodes, thymus, small intestine, bone marrow) and assessed the supernatants by ELISA for G-CSF production. Of all organs studied, G-CSF concentration was highest in bone marrow

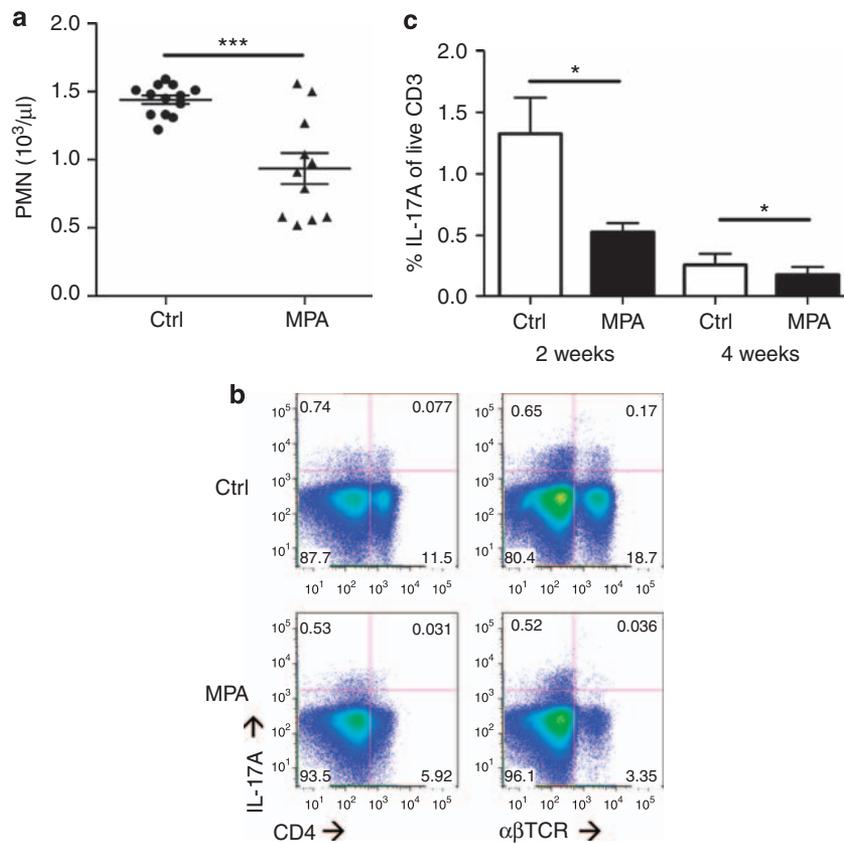


Figure 5 | Mycophenolate decreases neutrophil counts and IL-17-producing T cells during hematopoietic recovery. 7- to 9-week-old C57Bl/6 mice were lethally irradiated and transplanted with whole C57Bl/6 bone marrow. Circulating neutrophils in the MPA-treated (200 mg/kg/day) group (a) 2 weeks after transplantation ($n = 11-13$, three independent experiments) ($***P < 0.001$). IL-17-producing splenocytes were assessed by intracellular staining. A typical example at week 4 including CD4 and $\alpha\beta$ -TCR staining among all live splenocytes is shown in panel b. The percentage of IL-17-producing cells among all CD3⁺ cells was significantly decreased 2 and 4 weeks after transplantation (c, $n = 4-5$ at 4 weeks and 7-8 at 4 weeks from two independent experiments) ($*P < 0.05$).

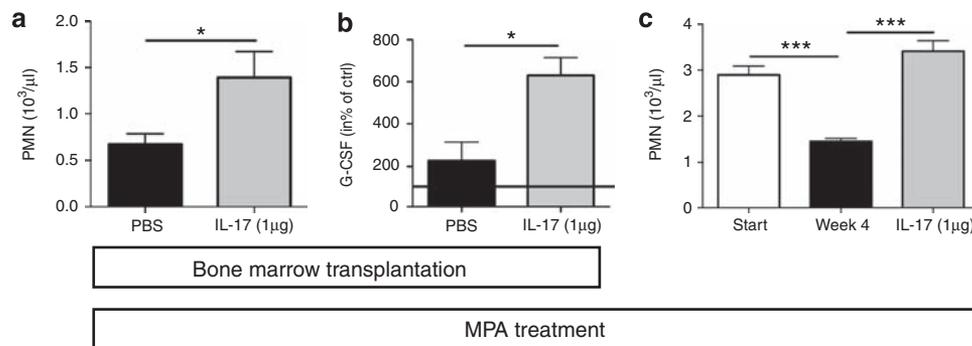


Figure 6 | Mycophenolate-induced decrease in neutrophil counts is rescued by IL-17. To test if IL-17A limited neutrophil recovery, mice were injected intraperitoneally with 1 μg recombinant IL-17A on day 10. Neutrophil counts (a, $n = 6-7$ from three independent experiments) and plasma G-CSF levels (b, $n = 3-4$, line: untreated controls) on day 14 were significantly increased by IL-17 treatment ($*P < 0.05$, $**P < 0.01$). To test if IL-17 replacement was also effective in mice treated with MPA without previous hematopoietic challenge, 8-week-old male C57Bl/6 mice were treated with MPA (200 mg/kg) for 4 weeks as in Figure 1c and received 1 μg recombinant IL-17A. Neutrophil counts were determined at the start, 4 weeks, and on day 3 after IL-17A injection (c, $n = 5$, $***P < 0.001$; Bonferroni after one-way analysis of variance).

supernatants (Figure 7a). To test if these G-CSF producers were sensitive to IL-17 stimulation, bone marrow was treated with IL-17 (50 $\mu\text{g}/\text{ml}$ for 5 h). This increased G-CSF secretion (Figure 7b). To investigate which cell type expressed the IL-17 receptor, bone marrow was analyzed

by multicolor flow cytometry. The highest IL-17 receptor expression was found on CD115⁺CD11b⁺ monocytes (Figure 7c). These results suggest bone marrow and monocytic cells as a relevant source of IL-17-dependent G-CSF production.

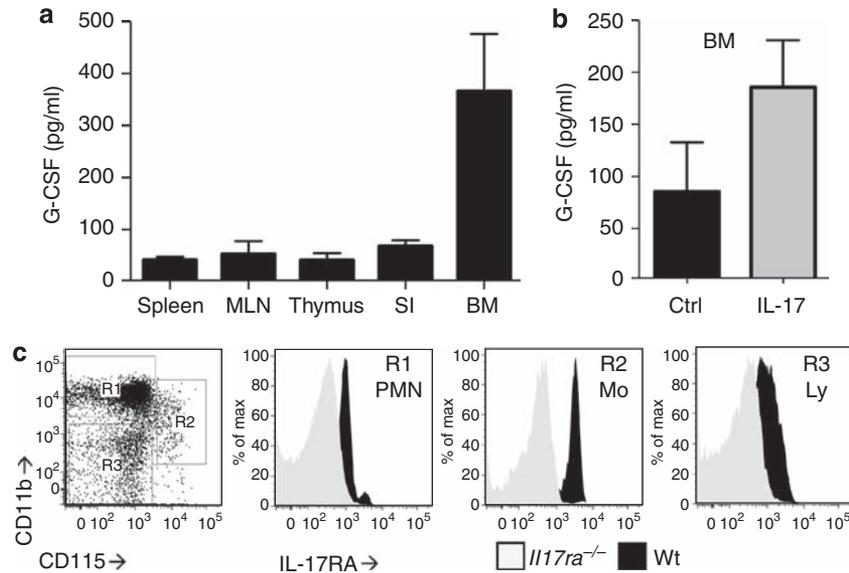


Figure 7 | The bone marrow secretes G-CSF in response to IL-17, and IL-17 receptor is highly expressed on bone marrow CD115⁺CD11b⁺ monocytes. Stimulation with PMA/ionomycin for 5 h and ELISA were used to determine the presence of potential G-CSF producers in a variety of tissues (a, $n = 3-7$). Production from bone marrow (BM) was higher than from spleen, mesenteric lymph nodes (MLN), thymus, and small intestine (SI). BM G-CSF production was increased by IL-17 (b, 50 $\mu\text{g/ml}$ for 5 h, $n = 5$). IL-17 receptor-A expression on BM cells was studied by flow cytometry. The highest expression was found on CD115⁺CD11b⁺ monocytes (c, a typical example from five wt animals, IL-17-receptor-deficient mice (*Il17ra*^{-/-}) served as control, gates were set to include neutrophils (PMN), monocytes (Mo), and lymphocytes (Ly)).

DISCUSSION

Our data show that MPA decreases circulating neutrophil counts in mice, MPA treatment suppresses T cell IL-17 production, IL-17 induces G-CSF production from bone marrow, and IL-17 substitution reverses MPA-induced decrease in peripheral blood neutrophil counts.

Neutropenia in MPA-treated patients is a typical dose-dependent and dose-limiting side effect. The late onset of neutropenia in patients, commonly after several months of treatment,^{2,57} suggested a mechanism distinct from direct myelotoxic effects of other agents such as cyclophosphamide or purine antagonists. We observed a decrease in circulating neutrophil counts in normal wt, but not *Il17ra*^{-/-} mice. This suggests that decrease in neutrophil counts by MPA is indeed an IL-17-mediated effect.

MPA acts on replicating, but not resting T cells.²³ Our data confirm a dose-dependent inhibition of T-cell proliferation. Renewal of all T cells is necessary after an acute event such as lethal irradiation and bone marrow transplantation, and a significant decrease of IL-17-producing T cells was observed in spleens of MPA-treated mice recovering from this event. However, T-cell renewal also occurs under steady-state conditions.⁵⁸ In addition and differential from classical T_{H1} and T_{H2} cells, IL-17 production is not only a terminal T-cell differentiation step but also a transient phenotype during T-cell development *in vivo*.⁵⁹⁻⁶¹ The generation of these transient IL-17 producers might also be susceptible to MPA. All mentioned mechanisms can reduce the amount of IL-17A available for induction of G-CSF production and thereby granulopoiesis.

Neutrophil counts in IL-17-receptor-deficient mice suggest that IL-17 is responsible for 40–50% of normal neutrophil counts in mice,³⁸ and other IL-17-independent mechanisms of G-CSF induction exist. One candidate among the T-cell-produced mediators is oncostatin-M, which has been reported to regulate hematopoietic progenitor cell homeostasis.⁶² As MPA-induced depression of circulating neutrophil counts was absent in *Il17ra*^{-/-} mice, this suggests that the oncostatin-M pathway may be of minor importance under the conditions studied here.

T-cell cytokine secretion is differentially affected by pharmacological inhibition of IMPDH. While IL-2 production remained unaffected, IFN- γ , IL-4, and IL-5 were significantly reduced by pharmacological inhibition *in vitro*.^{24,26} We explored the effects of MPA on IL-17 production, the signature cytokine of the recently defined T_{H17} lineage. Our data show strong inhibition of *in vitro* T_{H17} polarization induced by a combination of IL-6, IL-23, and TGF- β , and activating the T cells by anti-CD3 and anti-CD28 in mouse and human lymphocytes. This is similar to recent reports of IL-17 production in unpolarized human lymphocytes after 2–3 days,^{63,64} but not after 12-h short-term stimulation.⁶⁵ As IMPDHII activity is required for response to anti-CD3 and anti-CD28 stimulation,^{66,67} a decrease of IL-17 producer polarization using this mechanism is not unexpected. However, also under non-T_{H17}-specific conditions *in vitro* and during acute recovery from irradiation and bone marrow transfer *in vivo*, IL-17 production was reduced in relation to other T cells or their respective cytokines. These data raises the notion that IL-17 production may be more

sensitive to IMPDH inhibition than other T-cell cytokines, which will require closer investigation.

In addition to maintenance of neutrophil counts,^{37,38} IL-17 acts as an acute inflammatory cytokine. Its role has been shown in a broad range of autoimmune disease models, including different forms of arthritis, colitis, and experimental encephalitis.^{44,50,51} MPA is successfully used in a number of conditions that are associated with high IL-17 levels such as renal allograft rejection^{68–70} and cardiac allograft rejection characterized by massive neutrophil infiltration in T-bet-deficient mice.⁷¹ Very recently, IL-17 has also been implicated in immune-mediated renal disease. T_{H17} cells induced glomerulonephritis in response to antigens planted on the glomerular basement membrane⁷² and nephrotoxic nephritis.⁷³ IL-23 induces and stabilizes the population of IL-17-producing T cells.^{46,47} It was required for development of nephrotoxic glomerulonephritis and glomerulonephritis in response to immunization with the Good-pasture antigen.^{73,74} A role for IL-17 has also been shown in a mouse model of systemic lupus erythematosus, another condition successfully treated with MPA.^{15,16,75} Here, we exclusively study the role of MPA in regard to neutrophil homeostasis, but our results suggest the hypothesis that the benefit of MPA in autoimmune diseases and graft rejection is at least partially due to inhibition of T_{H17} cells.

Reversal of MPA-induced decrease in peripheral blood neutrophil numbers by IL-17 raises the question whether IL-17 is a possible intervention in MPA-induced neutropenia. From the role of IL-17 in autoimmune diseases, this appears counter-intuitive and would require extensive safety research. However, IL-17 application in an otherwise non-inflammatory setting was well tolerated during adenoviral overexpression³⁵ and injection of recombinant protein in our study. It is also of note that the pathogenicity of IL-17 very much depends on the cytokine context, especially IL-23⁵¹ and on the producing cell type, that is, CD4⁺ versus $\gamma\delta^+$ – or CD4⁺ $\alpha\beta^{\text{low}}$ T cells. As a much less experimental approach, G-CSF application as the mainstay of treatment in neutropenia has been successfully used in MPA-induced neutropenia in patients according to anecdotal reports.⁷⁶ In cases of neutropenia that are not anticipated, but already severe at detection, the value of G-CSF therapy has been questioned,⁷⁷ because endogenous G-CSF levels are elevated. If depression of granulopoiesis by MPA is due to lack of IL-17 and thereby G-CSF induction rather than direct myelotoxicity as our data suggest, G-CSF application is mechanistically more plausible and avoids the need to substitute IL-17.

Our data suggest that IL-17 levels might be investigated as an indicator of neutropenia risk during MPA treatment. While circulating IL-17 levels are below the detection limit of commonly used assays in normal mice,³⁸ they are detectable in normal human serum, albeit at low concentrations.^{70,78}

Our findings provide a mechanistic explanation of a common and treatment-limiting MPA side effect. This could open new avenues for risk assessment and possibly management of MPA-induced neutropenia.

MATERIALS AND METHODS

Animals

Wt C57Bl/6 mice (Jackson Labs, Bar Harbor, ME, USA) and mice lacking IL-17 receptor (*Il17ra*^{−/−}) (95% C57Bl/6 background)⁴⁰ were genotyped by PCR and used in age- and sex-matched groups. They were kept in specific pathogen-free conditions in a barrier facility. Animal experiments were approved by the Animal Care Committee at LIAI. Lethal irradiations were performed in a ¹³⁷Cesium irradiator (600 rad twice, 3 h apart) and mice were reconstituted with unfractionated wt bone marrow. Mycophenolate mofetil (Roche Pharma AG, Grenzach-Wyhlen, Germany) was incorporated into the chow (Research diets, New Brunswick, NJ, USA) at 300 mg/kg and 2 g/kg. This was calculated to reach an oral dose of 30 or 200 mg/kg body weight/day, respectively, at a chow consumption of 0.1 g/g. The measured chow consumption was 0.14 ± 0.02 g/g body weight per day in the low-dose and 0.15 ± 0.01 g/g body weight per day in the high-dose treatment and 0.15 ± 0.03 g/g body weight per day in the control groups (no significant differences). Recombinant IL-17A was from Peprotech (Rocky Hill, NJ, USA). Differential blood leukocyte counts were recorded using EDTA-coated capillary tubes, analyzed using an automatic analyzer (Hemavet 950; CDC Technologies, Oxford, CT, USA), and differential counts were confirmed manually (Sure stain, Fisher, Middletown, VA, USA).

Lymphocyte polarization, stimulation, staining, and flow cytometry

To induce IL-17 production, mouse total splenocytes or CD4⁺ cells sorted using the EasySep Mouse CD4⁺ T-cell-negative selection kit (Stemcell Technologies, Vancouver, BC, Canada), according to the manufacturer's instructions, were stimulated in complete medium (RPMI-1640 containing 10% fetal bovine serum, non-essential amino acids, 2 mM L-glutamine (all from Invitrogen, Carlsbad, CA, USA), and 1% penicillin/streptomycin) by plate-bound purified anti-CD28 and anti-CD3 (Biolegend, San Diego, CA, USA) in the presence of IL-6 (50 ng/ml), TGF- β (1 ng/ml; Peprotech), and IL-23 (20 ng/ml; eBioscience, San Diego, CA, USA) with the indicated concentrations of MPA with and without GMP (100 μ M) (both Sigma-Aldrich, Saint Louis, MO, USA) or dimethylsulfoxide-solvent control for 3 days. Human peripheral blood mononuclear cells were obtained by density-gradient centrifugation and cultured on anti-human CD28 and anti-CD3 (Biolegend, San Diego, CA, USA) using human IL-6 (50 ng/ml) and TGF- β (1 ng/ml; Peprotech). Before restimulation for flow cytometry or ELISA analysis, cells were transferred to an untreated plate and rested for 3 days. Polarized splenocytes and cell suspensions from spleen and thymus were stimulated for 5 h with 10 ng/ml PMA, 500 ng/ml calcium ionophore (both Sigma-Aldrich, Saint Louis, MO, USA), and 2 μ M monensin (eBioscience, San Diego, CA, USA). Anti-mouse-CD3 α -PerCP-Cy5.5 (145-2C11), anti-mouse-CD4-PerCP (RM4-5), anti-mouse-TCR β -APC (H57-597), anti-mouse IL-17A-PE (TC11-18H10.1), anti-mouse IL-17RA-PE (5G4), anti-mouse CD115-APC (AFS98), anti-mouse CD11b-Pacific blue (M1/70), anti-human IL-17A-PE (eBio64CAP17), and anti-human CD4-FITC (OKT4) (eBioscience and Biolegend, San Diego, CA, USA) were used with the LIVE/DEAD Fixable Dead Cell Stain kit (Invitrogen, Carlsbad, CA, USA) and BD-Fix-Perm (BD Pharmingen, San Jose, CA, USA). CFSE labeling was performed using the Celltrace CFSE cell proliferation kit (Invitrogen). Flow cytometry was performed using a Becton-Dickinson FACS Calibur or LSRII. Data were analyzed using the FlowJo software (Tree Star, Ashland, OR, USA). Gates for IL-17A were set using *Il17a*^{−/−} cells⁷⁹ (excluding 99.9% of cells).

mRNA quantification

RNA was isolated using Trizol (Invitrogen) and reverse transcribed using the Omniscript RT kit (Qiagen, Valencia, CA, USA) after DNase digest (RNase free DNase; Promega, Madison, WI, USA) according to the manufacturer's instructions. Real-time PCR was performed using the QuantiTect SYBR Green reverse transcription-PCR kit (Qiagen) using a Lightcycler 480 (Roche, Indianapolis, IN, USA). The primers for *Il17a* were selected using primer bank⁸⁰ as follows: TTTAACTCCCTGGCGCAAAA (forward), CTTTCCC TCCGCATTGACAC (reverse). Transcript levels were normalized to β -actin according to the ΔC_T method.

ELISA

Mouse IL-17A and G-CSF were assayed using Duo-Set ELISA development kits (R&D Systems, Minneapolis, MN, USA), IFN- γ and IL-5 using the mouse Th1/Th2 cytokine kit (BD Pharmingen), and human IL-17A using ELISA Ready-SET-Go (eBioscience) according to the manufacturer's instructions.

Statistical analysis

Two-tailed Student's *t*-test or paired *t*-test was used after analysis of variance if more than two values were compared. Data are expressed as mean \pm s.e.m.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure 1. Characteristics of MPA-treated mice.

Figure 2. Lymphocyte counts in mice treated with mycophenolate after bone marrow transplantation.

Figure 3. Neutrophil morphology and bone marrow characteristics in mycophenolate-treated mice after bone marrow transplantation.

Figure 4. Peripheral blood erythrocyte and thrombocyte counts in MPA-treated mice after bone marrow transplantation.

Figure 5. Lymphocyte and erythrocyte counts in IL-17-treated mice.

Supplementary material is linked to the online version of the paper at <http://www.nature.com/ki>

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